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# Characterization of Conserved Human Immunodeficiency Virus Type 1 gp120 Neutralization Epitopes Exposed upon gp120-CD4 Binding

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**Interaction with the CD4 receptor enhances the exposure on the human immunodeficiency type 1 gp120 exterior envelope glycoprotein of conserved, conformation-dependent epitopes recognized by the 17b and 48d neutralizing monoclonal antibodies. The 17b and 48d antibodies compete with anti-CD4 binding antibodies such as 15e or 21h, which recognize discontinuous gp120 sequences near the CD4 binding region. To characterize the 17b and 48d epitopes, a panel of human immunodeficiency virus type 1 gp120 mutants was tested for recognition by these antibodies in the absence or presence of soluble CD4. Single amino acid changes in five discontinuous, conserved, and generally hydrophobic regions of the gp120 glycoprotein resulted in decreased recognition and neutralization by the 17b and 48d antibodies. Some of these regions overlap those previously shown to be important for binding of the 15e and 21h antibodies or for CD4 binding. These results suggest that discontinuous, conserved epitopes proximal to the binding sites for both CD4 and anti-CD4 binding antibodies become better exposed upon CD4 binding and can serve as targets for neutralizing antibodies.**

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (4, 22). HIV-1 establishes a persistent infection in human hosts, eventually resulting in defective cellular immunity secondary to CD4 lymphocyte depletion (18, 41).

The HIV-1 exterior envelope glycoprotein, gp120, and the transmembrane envelope glycoprotein, gp41, are derived by cleavage of the gp160 envelope glycoprotein precursor (2, 67). HIV-1 is tropic for CD4-positive cells by virtue of a high-affinity interaction between the gp120 exterior envelope glycoprotein and the CD4 glycoprotein, which acts as the virus receptor (11, 38, 46, 49). Following gp120-CD4 binding, the fusion of viral and host cell membranes, which involves both gp120 and gp41 envelope glycoproteins, allows virus entry (28, 75). Amino acid changes in the third variable (V3) region of gp120, which forms a disulfide-linked loop (44), the fourth conserved (C4) gp120 region, the gp41 amino terminus, the gp41 ectodomain, or the gp41 transmembrane region dramatically reduce the efficiency of the membrane fusion process (13, 20, 21, 25, 28, 30, 33, 39, 40, 60, 77, 84).

Neutralizing antibodies appear to be an important component of a protective immune response (7, 16, 17, 24). HIV-1 neutralizing antibodies are directed against linear or discontinuous epitopes of the gp120 exterior envelope glycoprotein. Neutralizing antibodies that arise early in infected humans and that are readily generated in animals by immunization with gp120 or gp160 preparations are primarily directed against linear determinants in the V3 loop of the gp120 glycoprotein (35, 47, 48, 55, 64–66, 69, 71). These antibodies generally exhibit the ability to neutralize only a limited number of HIV-1 strains (23, 48, 57, 61), although a subset of anti-V3 antibodies recognize less variable elements of the region and therefore exhibit broader neutralizing activity (1, 24a, 34, 42, 58). Envelope glycoprotein variation within the linear V3 epitope and outside of the epitope can allow escape of viruses from neutralization by these antibodies (50, 56). These antibodies do not block CD4 binding but apparently interfere with post-receptor binding events involved in virus entry and syncytium formation, presumably by disrupting a step in the membrane fusion process (45, 72).

Later in the course of HIV-1 infection of humans, antibodies capable of neutralizing a wider range of HIV-1 isolates appear (6, 31, 53, 55, 64, 85). These broadly neutralizing antibodies have been difficult to elicit in animals (7, 27) and are not merely the result of additive anti-V3 loop reactivities against diverse HIV-1 isolates that accumulate during active infection (64). A subset of the broadly reactive antibodies, found in most HIV-1-infected individuals, interferes with the binding of gp120 and CD4 (36, 74). At least some of these antibodies recognize discontinuous gp120 epitopes present only on the native glycoprotein (26, 74). Antibodies that recognize conformation-dependent gp120 epitopes are highly prevalent in HIV-1-infected individuals (53). Human monoclonal antibodies derived from HIV-1-infected individuals have been identified that recognize the gp120 glycoproteins from a diverse range of HIV-1 isolates, that block gp120-CD4 binding, and that neutralize virus infection (31, 37, 63, 68, 82). Recently, the discontinuous epitopes recognized by five of these human monoclonal antibodies have been characterized (51, 78, 80). Amino acid changes in seven widely separated areas of the gp120 primary sequence, four of which overlap regions defined to be important for CD4 binding, disrupt recognition by these antibodies and, in some cases, allow the generation of neutralization escape mutants (51, 78, 80). The shared components of the antibody epitopes and the discontinuous CD4 binding region include threonine 257, aspartic acid 368,

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glutamic acid 370, lysine 421 to tryptophan 427, and aspartic acid 457 (10, 43, 51, 59, 78, 80). The anti-CD4 binding antibodies and anti-V3 loop antibodies exhibit additive or synergistic neutralization of HIV-1 (8, 79, 81).

The 17b and 48d monoclonal antibodies, which were derived from different HIV-1-infected individuals (67a), compete with each other and with the anti-CD4 binding antibodies for gp120 binding. These antibodies neutralize a number of divergent HIV-1 isolates, which suggests that they bind a well-conserved structure on the envelope glycoprotein (67a). Here we characterize the interaction of the 17b and 48d antibodies with the HIV-1 gp120 glycoprotein.

## MATERIALS AND METHODS

**Binding of radiolabeled anti-gp120 antibodies to transfected COS-1 cells.** COS-1 cells were transfected with 10 μg of pSVIIIenv DNA expressing wild-type or mutant HIV-1 envelope glycoproteins of the HXBc2 strain (28, 59). Sixty hours after transfection, the COS-1 cells were washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum and incubated with $^{35}$S-labeled monoclonal antibodies against gp120, either in the absence or in the presence of soluble CD4 (sCD4). Approximately $10^8$ antibody-producing hybridoma cells were labeled overnight in 60 to 80 ml of cysteine- and methionine-free RPMI medium containing 5 mCi each of [$^{35}$S]cysteine and [$^{35}$S]methionine (New England Nuclear). Nonlabeled cysteine and methionine were added in excess to the labeled medium. One milliliter of the antibody-containing medium was added to each 100-mm-diameter dish of transfected COS-1 cells. The envelope-expressing COS-1 cells were incubated with the antibody and, in some cases, with 1.3 μg of sCD4 (5, 12, 19, 32, 73, 83) per ml for 90 min at room temperature, washed three times with ice-cold PBS containing 2% fetal bovine serum, and lysed. Half of the lysates were incubated with protein A-Sepharose beads, which were washed according to the procedure used for immunoprecipitation (see below). The labeled antibody was visualized on a reducing sodium dodecyl sulfate (SDS)-polyacrylamide gel.

**Immunoprecipitations.** Immunoprecipitations were carried out as previously described (80, 86), using Nonidet P-40 (NP-40) buffer (0.5% NP-40, 0.5 M NaCl, 10 mM Tris HCl [pH 7.5]) to lyse the transfected COS-1 cells, after labeling with [$^{35}$S]cysteine. In some cases, sCD4 was added to the cell lysates to a final concentration of 10 μg/ml. Labeled cell lysates were precipitated at 4°C either with a mixture of sera from AIDS patients or with a particular monoclonal antibody (10 μg/ml, final concentration) and protein A-Sepharose beads. The precipitates were washed with NP-40 buffer and then analyzed on SDS-polyacrylamide gels. The relative recognition of mutant glycoprotein was estimated by visual inspection and, in some cases when decreases were observed, by densitometry.

**ELISA determination of mutant gp120 recognition by antibodies.** COS-1 cells were transfected with 10 μg of pSVIIIenv DNA expressing wild-type or mutant HXBc2 envelope glycoproteins. Sixty hours after transfection, cell supernatants were collected and frozen. For analysis of antibody recognition, the supernatants (100 μl), supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0), were incubated in wells of Immulon II enzyme-linked immunosorbent assay (ELISA) plates (Dynatech, Ltd.) coated with sheep antibody D7324 to the carboxyl-terminal 15 amino acids of gp120. Captured indirectly onto a solid phase in this way, gp120 retains the ability to bind sCD4 with high affinity (52). Monoclonal antibody 17b or 48d at a fixed concentration diluted in TMSS buffer (Tris-buffered saline [TBS] containing 2% nonfat milk powder and 20% sheep serum) was reacted with the captured gp120, and bound antibody was detected with alkaline phosphatase-conjugated goat anti-human immunoglobulin (Accurate Chemicals) and the AMPAK system (Dako Diagnostics) (53). The monoclonal antibody concentration was selected on the basis of titration curves to give approximately 75% of the level of binding to the wild-type gp120 at saturating antibody concentrations. Use of this antibody concentration resulted in signals of sufficient magnitude to be reliable while allowing sufficient sensitivity to small changes in the affinity of antibody for mutant glycoproteins.

Since the concentrations of the different mutant glycoproteins in the COS-1 supernatants differed and since normalization on a single wild-type well was too susceptible to random error, the binding of the monoclonal antibody to a particular mutant was compared with the binding to the entire panel of mutants. First, in quadruplicate plates, the reactivities of the gp120 mutants with a pool of HIV-1-positive sera were measured. These measurements were carried out in the presence of 0.5% Tween 20 to maximize reactivity with multiple epitopes and thus provide a measure of the amount of mutant protein available in the wells. After correction for background (using a mock-transfected COS-1 supernatant), the mean optical density at 492 nm ($OD_{492}$) value for each mutant was determined as a reference value. Second, the 17b or 48d antibody was assessed for binding to the mutant panel in duplicate or triplicate, and the mean $OD_{492}$ values were determined. For each mutant, the $OD_{492}$ ratio of monoclonal antibody to reference patient serum was calculated. The average value of this ratio (mean ± standard deviation) for the entire mutant panel was then calculated, eliminating from the calculation values near zero (i.e., mutants that do not bind antibody) to avoid skewing of the data. Amino acid changes that were scored as inhibitory to antibody binding exhibited ratios less than 0.5 times the mean ratio, which titration curves indicated represents a threefold or greater decrease in antibody affinity (data not shown and reference 51).

**Antibody competition analysis.** BH10 gp120 (25 ng/ml in TBS–10% fetal calf serum [FCS]) (Celltech, Ltd.; provided by the U.K. MRC AIDS Directed Programme) was captured onto a solid phase by antibody D7324 as described above. Competitor murine monoclonal antibodies were reacted with the captured protein for 30 min in 50 μl of TMSS buffer before addition of test antibody (48d) in 50 μl of TMSS buffer for a further hour. The amount of gp120-bound 48d antibody was determined by using alkaline phosphatase-conjugated goat anti-human immunoglobulin. Cross-reactivity of the goat antibody with the murine competitor antibodies was minimal and did not significantly interfere with the assays.

**Effect of sCD4 on antibody binding to monomeric gp120 glycoprotein.** BH10 gp120 (1 μg/ml in TBS–10% FCS) was incubated at room temperature for 45 min in the presence of 40 μg of sCD4 per ml and then diluted 20-fold in TBS–10% FCS to yield a final gp120 concentration of 50 ng/ml and a final sCD4 concentration of 2 μg/ml. One hundred microliters of the mixture was captured onto wells coated by antibody D7324 for 2 h. The wells were washed twice with TBS, and then different concentrations of monoclonal antibody in 100 μl of TMSS were incubated with the wells for 1 h. The wells were washed, and bound antibody was detected as described above.

**Virus neutralization assay.** Complementation of a single

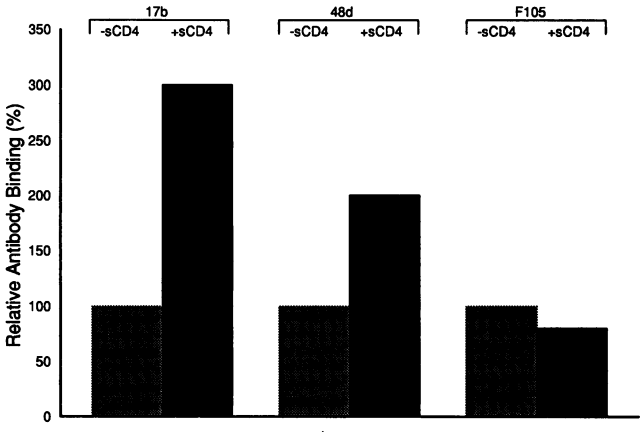


FIG. 1. Effect of sCD4 binding on recognition of cell surface HIV-1 envelope glycoproteins by monoclonal antibodies. COS-1 cells expressing wild-type HIV-1 envelope glycoproteins were incubated with radiolabeled antibody in the absence (grey bars) or presence (black bars) of 1.3 μg of sCD4 per ml. The values shown are those from a typical experiment; the amount of antibody bound was determined by densitometric scanning of the antibody heavy-chain band. Nonspecific antibody binding to COS-1 cells transfected with plasmid pSVIIIenvΔKS, which contains a deletion within the *env* gene, was subtracted from all values.

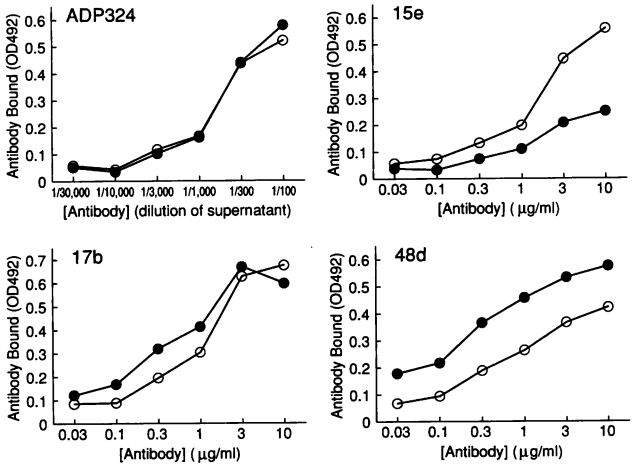


FIG. 2. Effect of sCD4 on antibody binding to monomeric gp120 glycoproteins. Binding of the ADP 324 (anti-V1/V2 region), 15e (anti-CD4 binding region), 17b, and 48d antibodies to captured HIV-1 gp120 glycoprotein is shown. The gp120 glycoprotein was preincubated in either the presence (●) or absence (○) of sCD4 prior to capture as described in Materials and Methods.

round of replication of the *env*-deficient chloramphenicol acetyltransferase (CAT)-expressing provirus by the mutant envelope glycoproteins was performed as previously described (28, 78, 80). For inhibition of virus replication, either no antibody or 30 μg of monoclonal antibody per ml was incubated with recombinant CAT-expressing virus for 1 h at 37°C before exposure of the virus to target Jurkat lymphocytes. The concentration of antibody was chosen to neutralize at least 80% of the virions containing wild-type HXBc2 envelope glycoproteins. CAT activity in the target cells was measured as previously described (28).

## RESULTS

**Enhanced recognition of HIV-1 envelope glycoproteins by the 17b and 48d antibodies following CD4 binding.** To examine the effect of CD4 binding on recognition of the HIV-1 envelope glycoproteins by the 17b and 48d antibodies, radiolabeled 17b and 48d antibodies were incubated in the absence or presence of sCD4 (5, 12, 19, 32, 73, 83) with COS-1 cells expressing the HIV-1 envelope glycoproteins (HXBc2 strain). An sCD4 concentration below that required to saturate the multimeric envelope glycoprotein complex on the cell surface and room temperature incubation were used to minimize gp120 shedding by sCD4 (27a, 51a). Figure 1 shows that incubation of envelope glycoprotein-expressing COS-1 cells at 37°C with 1.3 μg of sCD4 per ml resulted in three- and twofold increases in binding of the 17b and 48d antibodies, respectively. By contrast, binding of the F105 antibody, which recognizes an epitope overlapping the CD4 binding site (80), was slightly decreased by incubation of the COS-1 cells with the same concentration of sCD4. The relatively small effect on F105 binding indicates that under these conditions, gp120 shedding is minimal and only a small fraction of the available envelope glycoproteins are occupied by sCD4. F105 antibody binding was reduced by more than 90% when a higher concentration (35 μg/ml) of sCD4 was used in this assay (data not shown). Binding of labeled antibodies to COS-1 cells transfected with plasmid pSVIIIenvΔKS, which contains a deletion within the *env* gene, was significantly lower than that seen for the wild-type glycoprotein and was not enhanced by the presence of sCD4 (data not shown). These results demonstrate that sCD4 interaction with the HIV-1 envelope glycoproteins results in enhanced binding of the 17b and 48d antibodies to the envelope glycoprotein complex. Binding of the 48d antibody to monomeric, recombinant gp120 attached to ELISA plates via a carboxy-terminal antibody was also enhanced in the presence of added sCD4 (Fig. 2). Slight increases in binding of the 17b antibody to monomeric gp120 in the presence of sCD4 were also seen (Fig. 2). Thus, HIV-1 envelope glycoprotein oligomerization is not essential for the sCD4-induced enhancement of 48d or 17b binding. However, because of differences in the assays used herein, further studies will be required to accurately assess the relative contributions of monomer- and oligomer-specific interactions to 17b and 48d exposure.

**Effects of amino acid changes in the HIV-1 gp120 glycoprotein on antibody recognition.** The 17b and 48d antibodies recognize conformation-dependent epitopes on the HIV-1 gp120 envelope glycoprotein and compete with each other for binding gp120 (67a). Since these antibodies were raised in infected patients to random HIV-1 strains yet efficiently recognize HIV-1 IIIB and other laboratory HIV-1 isolates, the antibody epitopes must be reasonably well conserved among HIV-1 variants. To characterize these epitopes, the ability of the 17b and 48d antibodies to precipitate a panel of HIV-1 (HXBc2 strain) gp120 mutants from transfected COS-1 cell lysates was examined. Pilot experiments indicated that both 17b and 48d antibodies precipitated the gp160 and gp120 glycoproteins from COS-1 cell lysates prepared in NP-40 buffer. By contrast, almost no detectable precipitation of these proteins prepared in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 10 mM Tris HCl [pH 7.5]) was observed (data not shown), indicating that the 17b and 48d epitopes were either not well preserved or not exposed under these conditions. Thus, studies using enve-

lope glycoproteins in cell lysates were performed with NP-40 buffer. Since the studies described above indicated that CD4 binding could influence recognition of the HIV-1 envelope glycoproteins by these antibodies, precipitation of the envelope glycoproteins was performed in both the absence and presence of sCD4 in the COS-1 cell lysates.

The results of the immunoprecipitation studies are summarized in Table 1. Recognition of the HIV-1 envelope glycoproteins by the 17b and 48d antibodies in the absence of sCD4 was decreased by changes in several amino acids located in various regions of gp120. Significant decreases in envelope glycoprotein precipitation by both antibodies were observed for amino acid changes in C1 (Trp-69, Asn-88, and Lys-117), C2 (Arg-252, Thr-257, and Asn-262), C3 (Glu-370 and Gly-380 to Phe-382), C4 (Ile-420, Lys-421, Trp-427, Ala-433, Tyr-435, and Pro-438), and C5 (Met-475, Asp-477, and Lys-485). While single amino acid changes in the gp120 variable regions exhibited little effect on recognition by either antibody, neither the 17b nor 48d antibody precipitated HIV-1 gp120 mutants containing large deletions in the V1/V2 or V3 region (Δ119-205 and Δ297-329). Some mutants (183/184 PI/SG, 256 S/Y, 262 N/T, 266 A/E, 281 A/V, 384 Y/E, 421 K/L, and 485 K/V) were recognized differently by the 17b and 48d antibodies.

In contrast to the enhancing effect of sCD4 on recognition of the HIV-1 envelope glycoproteins by the 17b and 48d antibodies observed at room temperature in the absence of detergent, the presence of sCD4 did not significantly affect the ability of the wild-type glycoprotein to be precipitated at 4°C from NP-40 cell lysates by either antibody (data not shown). Similarly, all of the mutants efficiently precipitated by these antibodies in the absence of sCD4 were precipitated with equivalent efficiency in the presence of sCD4. By contrast, a number of envelope glycoprotein mutants that were inefficiently precipitated by the 17b or 48d antibody in the absence of sCD4 exhibited wild-type levels of recognition by these antibodies in the presence of sCD4. This phenomenon was observed for mutants containing single amino acid changes (69 W/L, 88 N/P, 113 D/R, 183/184 PI/SG, 207 K/W, 266 A/E, 281 A/V, 370 E/D, and 381 E/P) as well as for a mutant (Δ297-329) containing a large deletion of the V3 loop. Other mutants exhibiting low recognition by the 17b or 48d antibody in the absence of sCD4 exhibited some improvement in precipitation by these antibodies when sCD4 was present, but recognition in the latter case was still diminished compared with that seen for the wild-type glycoprotein. Finally, the low recognition of a number of mutants was not substantially improved by adding sCD4 to the lysate. In some cases, this lack of improved precipitation by antibody might be due to the poor ability of the mutant glycoprotein to bind sCD4 (e.g., 257 T/R, 370 E/R, 427 W/V, and 427 W/S). In other cases, the mutant glycoproteins were able to bind sCD4 (59), indicating that the poor recognition of the mutant by the antibody was not effectively altered by CD4 binding. Examples of the latter mutants include Δ119-205, which contains a deletion of the V1/V2 loop, and 257 T/A, 257 T/G, 421 K/L, 433 A/L, 470 P/G and 477 D/V.

**Recognition of HIV-1 gp120 mutants by the 17b and 48d antibodies in the absence of detergent.** Since the immunoprecipitations described above were carried out in the presence of NP-40, amino acids important for 17b or 48d recognition were studied in the absence of detergent. Because of the lability of the HIV-1 gp120-gp41 interaction, a fraction of the gp120 glycoprotein expressed in COS-1 cells is shed into the cell supernatant (29). This soluble gp120 was bound to ELISA plates by using an antibody directed to the gp120 carboxyl terminus (52). The reactivity of the bound gp120 mutants to the 17b or 48d antibody was measured, using a mixture of polyclonal sera from HIV-1-infected individuals to normalize the amount of mutant glycoprotein available for binding. With only a few exceptions, the amino acid changes identified as important for 17b or 48d precipitation of gp120 in NP-40-containing cell lysates were confirmed to be important for reactivity in the ELISA. The exceptions were observed for mutant 88 N/P, which was recognized efficiently by both 17b and 48d antibodies in the ELISA; the mutants involving changes in threonine 257, which in the ELISA were recognized efficiently by the 17b and 48d antibodies; mutant 421 K/L, which was recognized by the 17b antibody equivalently to the wild-type glycoprotein in the ELISA; and mutants 477 D/V and 485 K/V, which were recognized efficiently in the ELISA by both antibodies (data not shown).

In the ELISA format, it was noted that several amino acid changes (Δ119-205, 207 K/W, 381 G/F, 382 E/P, 384 Y/E, 420 I/R, 421 K/L, 427 W/V, 427 W/S, and 438 P/R) completely eliminated detectable 48d antibody recognition regardless of the antibody concentration used. For some gp120 mutants, however, use of increased concentrations of the 48d antibody allowed an increase in antibody binding to the mutant glycoprotein. This phenomenon was observed for mutants 69 W/L, 117 K/W, 252 R/W, 262 N/T, 433 A/L, and 475 M/S. These results suggest that the affinity of the 48d antibody-antigen interaction is affected less by the latter than by the former group of amino acid changes.

**Competition for gp120 binding between 48d and other monoclonal antibodies.** To complement the mutagenic analysis of the 48d epitope, several monoclonal antibodies were tested for the ability to compete with the 48d antibody for binding to HIV-1 gp120 from the BH10 strain immobilized on an ELISA plate as described above (52). As shown in Table 2, strong competition for gp120 binding was observed between the 48d antibody and antibody ICR 39.13g, which recognizes a discontinuous gp120 epitope overlapping the CD4 binding site (51). A moderate degree of competition was observed between the 48d antibody and the G3-42 and G3-508 antibodies (76), which recognize a linear C4 sequence (EVGKAMYAPP) between amino acids 429 and 438. The ADP 324 antibody, but not the ADP 325 antibody, also exhibited moderate competition for gp120 binding with the 48d antibody. Both ADP 324 and ADP 325 antibodies recognize determinants within the V1/V2 stem-loop structure (51b, 62). Other antibodies directed against the V1/V2 structure did not efficiently compete with the 48d antibody for gp120 binding. Weak or moderate competition was observed for some antibodies that recognize linear epitopes in the V3 gp120 region or in the C1 gp120 region between residues 60 and 90.

**Escape from neutralization by 17b and 48d antibodies.** To examine whether the amino acid changes that affected 17b or 48d antibody recognition in COS-1 cell lysates might alter sensitivity to neutralization, an *env* complementation assay was used (28). In this assay, recombinant virions are produced by cotransfection of COS-1 cells with a plasmid expressing the wild-type or a mutant envelope glycoprotein and a plasmid containing an *env*-deleted HIV-1 provirus encoding the CAT protein (28). The recombinant virions were incubated in the presence or absence of the 17b or 48d antibody prior to exposure to Jurkat target cells. The degree of neutralization was assessed by comparing the CAT activity in the target cells following exposure to viruses incubated with or without antibody.

TABLE 1. Precipitation of HIV-1 gp120 glycoprotein mutants in COS-1 cell lysates by 17b and 48d antibodies[a]

| gp120 region | Envelope glycoprotein | Recognition by monoclonal antibody: | | | |
|---|---|---|---|---|---|
| | | 17b | | 48d | |
| | | Without sCD4 | With sCD4 | Without sCD4 | With sCD4 |
| | Wild type | ++++ | ++++ | ++++ | ++++ |
| C1 | 36 V/L | ++++ | ++++ | ++++ | ++++ |
| | 40 Y/D | ++++ | ++++ | ++++ | ++++ |
| | 45 W/S | ++++ | ++++ | ++++ | ++++ |
| | 69 W/L | ++ | ++++ | ++ | ++++ |
| | 76 P/Y | ++++ | ++++ | ++++ | ++++ |
| | 80 N/R | ++++ | +++ | ++++ | ++++ |
| | 88 N/P | + | ++ | ++ | ++++ |
| | 102 E/L | ++++ | ++++ | ++++ | ++++ |
| | 103 Q/F | +++ | ++++ | ++++ | ++++ |
| | 106 E/A | ++++ | ++++ | ++++ | ++++ |
| | 113 D/A | +++ | ++++ | ++++ | ++++ |
| | 113 D/R | ++ | ++++ | ++ | ++++ |
| | 117 K/W | + | ++ | ++ | +++ |
| | 120/121 VK/LE | +++ | +++ | +++ | +++ |
| | 125 L/G | ++++ | ++++ | ++++ | ++++ |
| V1/V2 | 152/153 GE/SM | ++++ | ++++ | ++++ | ++++ |
| | 166 R/L | ++++ | ++++ | +++ | ++++ |
| | 168 K/L | ++++ | ++++ | ++++ | ++++ |
| | 176/177 FY/AT | ++++ | ++++ | ++++ | ++++ |
| | 179/180 LD/DL | ++++ | ++++ | ++++ | ++++ |
| | 183/184 PI/SG | ++ | ++++ | ++++ | ++++ |
| | 191/192/193 YSL/GSS | +++ | ++++ | ++++ | ++++ |
| | Δ119-205 | − | + | − | + |
| C2 | 207 K/W | − | +++ | − | ++++ |
| | 252 R/W | ++ | +++ | ++ | +++ |
| | 256 S/Y | ++++ | +++ | − | + |
| | 257 T/R | ++ | ++ | ++ | ++ |
| | 257 T/A | ++ | ++ | ++ | ++ |
| | 257 T/G | + | ++ | ++ | ++ |
| | 262 N/T | ++ | +++ | − | + |
| | 266 A/E | ++ | ++++ | ++++ | ++++ |
| | 267 E/L | +++ | +++ | +++ | +++ |
| | 269 E/L | ++++ | ++++ | ++++ | ++++ |
| | 281 A/V | ++ | ++++ | ++++ | ++++ |
| V3 | 298 R/G | +++ | +++ | ++ | +++ |
| | 308/309/310 RIQ/RPELIPVQ | ++++ | ++++ | ++++ | ++++ |
| | 313 P/S | ++++ | ++++ | ++++ | ++++ |
| | 314 G/W | +++ | ++++ | ++++ | ++++ |
| | Δ297-329 | − | ++++ | − | ++++ |
| | 356 N/I | ++++ | ++++ | ++++ | ++++ |
| C3 | 368 D/R | ++++ | ++++ | ++++ | ++++ |
| | 368 D/P | ++++ | ++++ | ++++ | ++++ |
| | 368 D/T | ++++ | ++++ | ++++ | ++++ |
| | 368 D/K | ++++ | ++++ | +++ | +++ |
| | 370 E/Q | + | ++ | ++ | ++ |
| | 370 E/R | ++ | ++ | ++ | ++ |
| | 370 E/D | ++ | ++++ | + | +++ |
| | 380 G/F | + | ++++ | + | +++ |
| | 380/381 GE/FY | − | ++ | − | + |
| | 381 E/P | − | ++++ | − | +++ |
| | 382 F/L | + | ++ | + | ++ |
| | 384 Y/E | ++++ | ++++ | + | ++++ |
| V4 | 386 N/Q | ++++ | ++++ | ++++ | +++ |
| | 392 N/E 397 N/E | ++++ | ++++ | ++++ | ++++ |
| | 395 W/S | ++++ | ++++ | ++++ | ++++ |
| | 406 N/G | ++++ | ++++ | ++++ | ++++ |
| C4 | 420 I/R | − | + | − | ++ |
| | 421 K/L | ++ | ++ | − | + |
| | 427 W/V | − | − | − | − |
| | 427 W/S | − | − | − | − |
| | 429 K/L | ++++ | ++++ | ++++ | ++++ |
| | 430 V/S | ++++ | ++++ | ++++ | ++++ |

*Continued on following page*

TABLE 1—*Continued*

| gp120 region | Envelope glycoprotein | Recognition by monoclonal antibody: 17b Without sCD4 | 17b With sCD4 | 48d Without sCD4 | 48d With sCD4 |
|---|---|---|---|---|---|
| | 432 K/A | +++ | +++ | ++++ | ++++ |
| | 433 A/L | ++ | ++ | ++ | +++ |
| | 435 Y/H | +++ | +++ | ++++ | ++++ |
| | 435 Y/S | − | ++ | + | ++ |
| | 438 P/R | ++ | +++ | + | +++ |
| | 450 T/N | ++++ | ++++ | ++++ | ++++ |
| | 456 R/K | ++++ | ++++ | ++++ | ++++ |
| | 457 D/A | +++ | +++ | ++++ | ++++ |
| | 457 D/G | ++++ | +++ | +++ | +++ |
| | 457 D/R | +++ | ++ | +++ | +++ |
| | 457 D/N | ++++ | ++++ | ++++ | ++++ |
| | 457 D/E | ++++ | +++ | +++ | ++++ |
| V5 | 463 N/D | ++++ | ++++ | ++++ | ++++ |
| C5 | 470 P/G | ++ | ++ | +++ | +++ |
| | 475 M/S | − | ++ | + | ++ |
| | 477 D/V | + | ++ | ++ | ++ |
| | 485 K/V | + | ++ | +++ | +++ |
| | 491 I/F | ++++ | ++++ | ++++ | ++++ |
| | 493 P/K | ++++ | ++++ | ++++ | ++++ |
| | 495 G/K | ++++ | +++ | +++ | +++ |
| | 497/498/499 APT/VLL | ++++ | ++++ | ++++ | ++++ |
| | 500/501 KA/KGIPKA | +++ | +++ | +++ | +++ |

[a] Recognition of the mutant glycoproteins by each monoclonal antibody relative to recognition of the wild-type glycoprotein was assessed by precipitation. The amounts of mutant and wild-type glycoproteins present in the lysate were assessed by a parallel precipitation using a mixture of sera from HIV-1-infected individuals. Symbols: ++++, precipitation approximately equivalent to that of the wild-type glycoprotein; +++, signals 61 to 90% of the wild-type level; ++, signals 16 to 60% of the wild-type level; +, signals 1 to 15% of the wild-type level; −, no precipitation.

Table 3 shows the ratios of CAT activity in Jurkat target cells detected after exposure of recombinant viruses to 30 μg of 17b or 48d antibody per ml at 37°C for 1 h compared with the activity seen when recombinant virions were similarly incubated without antibody. This concentration of antibody neutralized more than 80% of recombinant viruses containing the wild-type HIV-1 envelope glycoprotein. The sensitivity of several mutants to neutralization by both antibodies was diminished compared with that of virus containing the wild-type glycoprotein. Although the efficiency of virus entry varied among the different envelope glycoproteins, this value did not demonstrate any obvious relationship to neutralization sensitivity. Significant decreases in sensitivity to neutralization by both the 17b and 48d antibodies were found for mutants 113 D/R, 252 R/W, 257 T/A, 257 T/G, 370 E/D, 382 F/L, 420 I/R, 433 A/L, 438 P/R, and 475 M/S. Mutant 421 K/L was the only envelope glycoprotein tested that exhibited a significant difference between 17b and 48d neutralization. Mutant 421 K/L was neutralized efficiently by the 17b antibody but not by the 48d antibody.

## DISCUSSION

For viruses like HIV-1 that fuse viral and target cell membranes at neutral pH, receptor binding is thought to induce conformational changes in the envelope glycoproteins necessary for the fusion event (3, 40, 54). The 17b and 48d epitopes on the HIV-1 gp120 envelope glycoprotein are present on the native envelope glycoprotein complex in the absence of sCD4 but are increased in number or accessibility by sCD4 binding. These results suggest that CD4 binding can induce conformational changes in the HIV-1 envelope glycoprotein complex. That some enhancement of 48d and 17b antibody binding to a monomeric gp120 protein in ELISA occurred indicates that at least part of the gp120 conformational change can occur within a single polypeptide chain and that a change in accessibility probably underlies this

TABLE 2. Competition of anti-gp120 monoclonal antibodies with the 48d antibody for binding to monomeric gp120

| Competing antibody | Concn of competing antibody used[a] | HIV-1 gp120 region recognized | % 48d antibody bound ± SD |
|---|---|---|---|
| None | | | 100 ± 11 |
| ICR 39.13g | 1/500 | Discontinuous CD4 binding site | 5 ± 2 |
| G3-42 | 2 | C4 | 35 ± 4 |
| G3-508 | 2 | C4 | 33 ± 5 |
| ADP 324 | 1/100 | V1/V2 | 44 ± 8 |
| ADP 325 | 1/20 | V1/V2 | 92 ± 8 |
| G3-4 | 2 | V2 | 92 ± 14 |
| G3-136 | 5 | V2 | 101 ± 8 |
| BAT-085 | 10 | V2 | 93 ± 6 |
| 52-581-SC258 | 10 | V1/V2 | 70 ± 7 |
| 52-684-238 | 1 | V1/V2 | 89 ± 7 |
| BAT-123 | 1 | V3 | 59 ± 9 |
| 9284 | 5 | V3 | 55 ± 10 |
| 110.5 | $1/10^4$ | V3 | 85 ± 11 |
| 110.J | 10 | V3 | 41 ± 11 |
| 110.4 | $1/10^4$ | V3 | 71 ± 3 |
| 133/290 | 10 | C1 (residues 60–72) | 55 ± 8 |
| ADP 360 | 1/100 | C1 (residues 80–90) | 49 ± 6 |
| B2 | 10 | C1 (residues 90–100) | 82 ± 10 |

[a] Saturating concentrations of competing antibodies, determined in pilot experiments, were used in all assays. Antibody concentrations are reported as micrograms of purified antibody per milliliter or as dilutions of hybridoma supernatants.

phenomenon. The importance of this or other reported CD4-induced conformational changes in the HIV-1 envelope glycoproteins (9, 70) for the function of the glycoproteins is still unknown. The targeting of the 17b and 48d epitopes by antibodies able to neutralize virus infection makes these attractive candidates for functionally relevant conformational alterations.

Recognition by the 17b and 48d antibodies is markedly sensitive to changes in gp120 conformation. Neither the gp120 nor the gp160 glycoprotein was precipitated by these antibodies under the conditions present in RIPA lysis buffer. A large fraction of the amino acid changes tested in this study affected envelope glycoprotein recognition by both antibodies. Three approaches were used to identify gp120 regions that are most important for antibody recognition under a variety of conditions. First, the precipitation of the HIV-1 envelope glycoproteins in cell lysates was carried out in the presence of sCD4, which restored antibody binding to a number of mutant glycoproteins. This finding suggests that sCD4 can restrict the conformational flexibility that results from some amino acid changes and thereby improve the efficiency of antibody recognition. Residues in which changes resulted in decreased recognition both in the absence and in the presence of sCD4 are more likely to contribute significantly to the antigen-antibody interaction. Second, gp120 recognition by the 17b and 48d antibodies was examined both in detergent-containing cell lysates and in cell supernatants in the absence of detergent. Soluble envelope glycoproteins present in cell supernatants have been selected for the ability to be transported through the endoplasmic reticulum and Golgi complex, a process dependent on proper glycoprotein folding (14, 15). Third, the replication-competent subset of mutants was examined for sensitivity to neutralization. Since the conformation of the envelope glycoproteins required for virus entry must approximate that of the wild-type glycoproteins, neutralization escape studies identify changes that directly affect antibody-gp120 interaction without a global disruption of gp120 conformation. Finally, a significant amount of information relevant to an assessment of conformational correctness has already been obtained for this mutant panel. This information includes rate of gp160 precursor processing, CD4 binding ability, gp120-gp41 association, and recognition by other conformation-dependent antibodies (29, 59, 77).

Given these considerations, five discontinuous, conserved HIV-1 gp120 elements are likely to contribute to the 17b and 48d epitopes or to reside close enough to the epitopes to influence antibody binding by local conformational effects. One element is the hydrophobic ring-like structure formed by a pair of disulfide bonds that link C3 and C4 (44). A second set of elements is contributed by the amino acids located at the base of a stem-loop structure (44) that includes the V1 and V2 variable regions of the gp120 glycoprotein. While the V1 and V2 regions located in the loops exhibit significant variability among HIV-1 strains, the stem structure apparently important for 17b and 48d recognition is well conserved. The conserved, hydrophobic C2 region extending from arginine 252 to asparagine 262 apparently contributes to the 17b and 48d epitopes. Changes in the more polar glutamic acid at 370 affected the efficiency of both 17b and 48d binding and neutralization. Finally, the binding of both antibodies was affected by a change in methionine 475, in the C5 gp120 region. The generally hydrophobic nature of the mapped components is consistent with the relatively limited exposure of the 17b and 48d epitopes on the native glycoprotein complex in the absence of CD4.

The epitopes of the 17b and 48d antibodies mapped by these techniques were very similar, with only slight quantitative differences in the effects of most amino acid changes on the antibody-gp120 interaction. One qualitative difference in the binding sites of the two antibodies is reflected in the observation that in the soluble gp120 recognition assay and in the neutralization escape assay, the 421 K/L change affects the 48d-gp120 interaction significantly more than the 17b-gp120 interaction.

The characterization of the epitopes of the 17b and 48d antibodies provides some insight into the behavior of these antibodies with respect to CD4 and to other monoclonal antibodies (Fig. 3). Both 17b and 48d epitopes were affected by changes in glutamic acid 370 and tryptophan 427, which make important contributions to CD4 binding (10, 59). Alterations in the regions surrounding these residues following CD4 binding might account for altered accessibility of the 17b and 48d epitopes. The 17b and 48d cross-competition for gp120 binding with the anti-CD4 binding antibodies (67a) (Table 2) can be explained by the importance of threonine 257 and glutamic acid 370 for recognition by both sets of antibodies (51, 78, 80). Some of the anti-CD4 binding antibodies also depend on amino acids in C4 near lysine 421 and tryptophan 427, in C5 near methionine 475, and in C1 near aspartic acid 113 and lysine 117 (51, 78, 80), all of which appear to be in proximity to the 17b and 48d epitopes. In this study, moderate competition for gp120 binding was observed between the 48d antibody and antibodies (76) directed against an exposed linear C4 sequence extending from

TABLE 3. CAT activity in Jurkat target cells

| Envelope glycoprotein | Inhibited/uninhibited CAT activity (%)[a] | |
|---|---|---|
| | 17b | 48d |
| Wild type | 17 | 11 |
| 80 N/R | 37 | 27 |
| 103 Q/F | 17 | 17 |
| 106 E/A | 22 | 22 |
| 113 D/R | 95 | 76 |
| 117 K/W | 50 | 60 |
| 120/121 VK/LE | 15 | 35 |
| 252 R/W | 67 | 80 |
| 257 T/A | 63 | 60 |
| 257 T/G | 66 | 62 |
| 266 A/E | 35 | 24 |
| 267 E/L | 18 | 12 |
| 281 A/V | 14 | 14 |
| 298 R/G | 10 | 12 |
| 313 P/S | 15 | 11 |
| 368 D/T | 11 | 11 |
| 370 E/Q | 50 | ND |
| 370 E/D | 82 | 102 |
| 382 F/L | 60 | 50 |
| 420 I/R | 100 | 83 |
| 421 K/L | 9 | 86 |
| 432 K/A | 61 | ND |
| 433 A/L | 62 | 59 |
| 438 P/R | 58 | 92 |
| 457 D/A | 15 | 11 |
| 470 P/G | 29 | 14 |
| 475 M/S | 97 | 100 |
| 477 D/V | 13 | 13 |
| 485 K/V | 40 | 35 |

[a] Ratio of CAT activity (as a percentage) observed in Jurkat lymphocytes incubated with virus plus monoclonal antibody compared with that observed for cells incubated with untreated virus. ND, not determined.

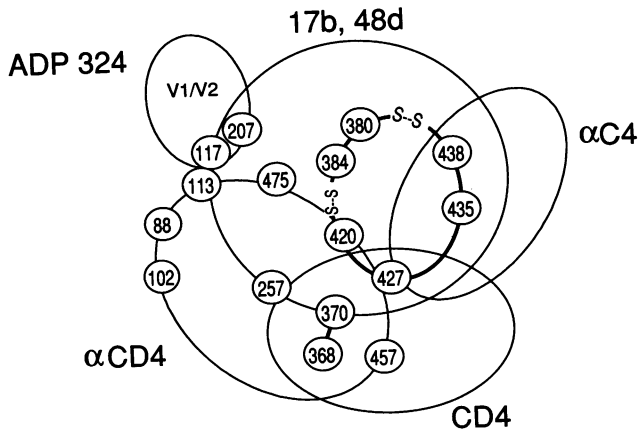


FIG. 3. Schematic diagram of HIV-1 gp120 residues important for binding CD4 or antibodies. The amino acid residues in which changes affect binding of CD4, anti-CD4 binding antibodies (αCD4), the 17b and 48d antibodies, the ADP 324 antibody, or antibodies directed against a linear C4 epitope encompassing residues 429 to 438 (αC4) are shown in the appropriate circles. Residues that are not completely included within a region indicate amino acids in which only some changes affect binding (or, in the case of the anti-CD4 binding antibodies, in which changes affect only some members of the group). The indicated overlap between the CD4 and 17b/48d binding sites on gp120 should not be interpreted to indicate competition between CD4 and the 17b/48d antibodies, but only that changes in these residues can affect both CD4 and 17b/48d binding. The presence of disulfide bonds (S– –S) linking the C3 and C4 gp120 regions into a hydrophobic ring is indicated.

residues 429 to 438. This finding is consistent with the observation that amino acid changes in this region diminished 17b and 48d recognition and resulted in decreased sensitivity to neutralization by these antibodies. Likewise, the ADP 324 antibody competed with the 48d antibody for binding to gp120. The ADP 324 antibody recognizes a conformation-dependent epitope within the V1/V2 region (51b), but the exact epitope recognized by this antibody is unknown. Recognition of the HIV-1 gp120 glycoprotein by ADP 324 is disrupted by the same amino acid changes near the base of the V1/V2 stem that affect 17b and 48d antibody recognition (51b), suggesting that this conserved structure may be proximal to both the ADP 324 and the 17b and 48d epitopes.

Further work will be necessary to understand the functional significance of the CD4-induced conformational changes in the gp120 glycoprotein affecting 17b and 48d antibody recognition. It is interesting that the 17b and 48d epitopes appear to contain components of the CD4 binding site and the ring-like structure formed by C3 and C4, which has been shown to contribute to the membrane fusion process and to the affinity of the gp120-gp41 interaction (29, 77). Exposure of the 17b and 48d epitopes may reflect CD4-induced alteractions in the gp120-gp41 interaction important for subsequent events leading to membrane fusion. In addition to promoting understanding of the virus entry process, knowledge of conserved HIV-1 neutralizing antibody epitopes may help to define methods for effectively generating prophylactic or therapeutic antiviral immune responses.

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